U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

STUDY OF *EWSR1* REARRANGEMENT IN THYROID CARCINOMA

CARLOS GUILHERME SANTOS DE OLIVEIRA

DISSERTAÇÃO DE MESTRADO EM ONCOLOGIA

2015

CARLOS GUILHERME SANTOS DE OLIVEIRA

STUDY OF *EWSR1* REARRANGEMENT IN THYROID CARCINOMA

Dissertação de candidatura ao grau de Mestre em Oncologia - especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

ORIENTADOR:
Professora Doutora Catarina Eloy
Instituto de Patologia e Imunologia Molecular da Universidade do Porto – Ipatimup, Porto, Portugal
Faculdade de Medicina da Universidade do Porto, Porto, Portugal

ACKNOWLEDGEMENTS

O meu maior agradecimento não podia deixar de ser para a minha orientadora, Prof. Doutora Catarina Eloy, pela oportunidade de trabalhar com ela ao longo de um ano num projeto ambicioso e com grandes perspetivas futuras. Agradeço ainda todo o apoio, disponibilidade, confiança, dedicação e amizade que sempre teve para comigo e para com o trabalho executado por mim. Foi com ela que mais aprendi e evoluí profissionalmente e pessoalmente, pois mais importante que o trabalho desenvolvido foi a relação de amizade existente, que cresceu ao longo de todo este caminho.

Agradeço à Técnica Dina Leitão por ter ajudado ao longo do projeto no que diz respeito a todas as burocracias referentes a encomendas de material e por ter deixado usufruir do laboratório e todos os equipamentos necessários para executar as tarefas práticas.

Não podia deixar de dizer o meu obrigado ao Dr. António Polónia por me apoiar e aconselhar a tomar as melhores decisões no que diz respeito à "estatística", e ainda por conversar comigo nos momentos chave.

Agradeço ao Prof. Doutor Sobrinho Simões por ser um exemplo para todos nós e por ter sempre algo a acrescentar, com o objetivo de melhorar aquilo já feito.

Às minhas "camaradas" de laboratório agradeço o ambiente que proporcionaram, tal como os momentos de lazer apesar de todo o trabalho existente. A entreajuda, amizade, companheirismo e boa disposição.

Devo também um enorme agradecimento a duas pessoas, as quais não preciso de dizer o nome para saberem quem são, por sempre me terem recebido em sua casa, por me proporcionarem momentos divertidos de amizade, jantares e brincadeiras, e acima de tudo por serem mais uma família para mim.

Queria ainda deixar um enorme agradecimento à Li, por sempre me apoiar, ouvir, incentivar, compreender, confortar e esperar sempre pelo melhor de mim, mesmo quando eu próprio não acreditava que seria capaz.

Ao meu irmão agradeço a sua boa disposição e admiração que faz com que eu, mesmo nas alturas de desânimo, tenha um sorriso na cara e acredite que o amanhã é melhor.

Por fim mas não menos importantes, fica um grande agradecimento aos meus pais, pois mais do que familiares têm sido os meus grandes pilares, que me apoiam e me fazem crescer a cada dia que passa, fazendo de mim uma pessoa melhor, mais humana e com a crença que irei alcançar tudo o que mais desejo.

TABLE OF CONTENTS

Figure índex VII

Table índex IX

Relevant abbreviations XI

Summary XIII

Resumo XV

1 - Introduction 1

1.1 - Thyroid general aspects 3

1.2 - Primary small cell tumours of the thyroid 4

1.2.1 - Primary extraeskeletal Ewing family tumour (PEEFT) 4

1.2.2 - Carcinoma of the thyroid with Ewing family tumour elements (CEFTE) 6

1.3 - Papillary thyroid carcinoma (PTC) 6

1.4 - Solid cell nests (SCN) 9

2 - Aim of the study 11

3 - Materials and methods 15

3.1 - Biological samples 17

3.2 - Fluorescence *in situ* hybridization (FISH) 17

3.3 - Statistic analysis 19

4 - Results 21

4.1 - Carcinoma of the thyroid with Ewing family tumour elements (CEFTE) 23

4.2 - Papillary thyroid carcinoma (PTC) 24

4.3 - Solid cell nests (SCN) 29

5 - Discussion 31

6 - Conclusion and future perspectives 37

6.1 - Conclusion 39

6.2 - Future perspectives 39

7 - References 41

8 - Annexes 49

FIGURE INDEX

Figure 1 - Localization of thyroid gland....3

Figure 2 - Metabolism of thyroid gland 3

Figure 3 - Histological aspect of PEEFT 5

Figure 4 - Diagrammatic representation of the *EWSR1-FLI1* rearrangement 5

Figure 5 - Histological aspect of CEFTE 6

Figure 6 - Histological aspect of classic PTC 8

Figure 7 - Histological aspect of SCN 9

Figure 8 - Imunohistochemistry and FISH analysis of CEFTE....23

Figure 9 - FISH analysis of PTC....26

Figure 10 - FISH analysis of SCN 29

TABLE INDEX

Table 1 - Differential diagnosis of thyroid tumours with neuroendocrine differentiation 4

Table 2 - Clinico-pathological features of the 54 PTC cases.....24

Table 3 - FISH analysis of the 54 PTC cases 26

Table 4 - Statistic analysis of clinico-pathological features and results of the FISH analysis of PTC cases 28

Table 5 - Clinico-pathological features and results of the FISH analysis of SCN cases ..29

RELEVANT ABBREVIATIONS

ATC	Anaplastic thyroid carcinoma
BRAF	B-Raf proto-oncogene
CD99	Cluster of differentiation 99
CEA	Carcinoembryonic antigen
CEFTE	Carcinoma of the thyroid with Ewing family tumour elements
ES	Ewing sarcoma
EWSR1	Ewing sarcoma breakpoint region 1
FISH	Fluorescence *in situ* hybridization
FLI1	Friend leukemia virus integration site 1
FNAB	Fine needle aspiration biopsy
HCl	Hydrochloric acid
HT	Hashimoto's thyroiditis
NTRK1	Neurotrophic tyrosine kinase receptor 1
PDTC	Poorly differentiated thyroid carcinoma
PEEFT	Primary extraeskeletal Ewing family tumour
PTC	Papillary thyroid carcinoma
RAI	Radioactive iodine
RET	Tyrosine kinase receptor gene
SCN	Solid cell nest
T_3	Triiodothyronine
T_4	Thyroxine
TG	Thyroglobulin
TK	Tyrosine kinase
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone
TTF-1	Thyroid transcription factor 1

SUMMARY

Carcinomas of the thyroid with Ewing family tumour element (CEFTEs) are small cell thyroid tumours with epithelial differentiation that co-exist with papillary thyroid carcinoma (PTC) foci, disclose p63 expression, *EWSR1-FLI1* rearrangement and likely a favorable prognosis. Two histogenetic hypotheses have been advanced regarding the origin of CEFTEs: arising in PTCs or in solid cell nests (SCN).

A total of 3 CEFTEs, 54 PTCs and 10 SCNs were collected from March 2011 to February 2015 and the morphological features of each case were reviewed by two pathologists. Fluorescence *in situ* hybridization (FISH) technique was performed in sections of all cases obtained from paraffin embedded tissue to search for the presence of *EWSR1* rearrangements.

All 3 CEFTEs disclosed the *EWSR1-FLI1* rearrangement both in the small cell and in the PTC components. Out of the 54 PTC cases, 28 (51.9%) were positive, 20 (37.0%) were negative and 6 (11.1%) were inconclusive for *EWSR1* rearrangement; in 2 of the positive PTC cases the *EWSR1-FLI1* rearrangement was detected. Classic PTC disclosed more often the *EWSR1* rearrangement than other PTC variants (p=0.031). PTCs with *EWSR1* rearrangement disclosed less often nuclei with *EWSR1* polysomy than those without *EWSR1* rearrangement (p=0.001). Out of the 10 SCNs, 7 (70.0%) were negative and 3 (30.0%) were inconclusive for the *EWSR1* rearrangement. Monosomic nuclei were more frequent (mean of 44.3%) in SCNs than in PTCs (p<0.001).

The presence of the *EWSR1-FLI1* rearrangement in PTC component of all studied CEFTEs and the existence of the *EWSR1* rearrangement in some PTCs favours the origin of CEFTE from PTC. The high frequency of *EWSR1* rearrangements in PTC may represent a new diagnostic marker of these tumours.

Keywords: Papillary thyroid carcinoma, *EWSR1* rearrangement, Small cell carcinoma, *EWSR1-FLI1*, Ewing sarcoma.

RESUMO

Carcinomas da tiroide com elementos de tumores da família Ewing (CEFTEs) são tumores da tiroide de pequenas células com diferenciação epitelial que coexistem com focos de carcinoma papilar da tiroide (PTC), expressam p63, rearranjo *EWSR1-FLI1* e provavelmente um prognóstico favorável. Duas hipóteses histogenéticas para a origem dos CEFTEs têm sido avançadas: origem em PTCs ou em *solid cell nests* (SCN).

Um total de 3 CEFTEs, 54 PTCs e 10 SCNs foram selecionados desde março de 2011 até fevereiro de 2015 e as características morfológicas de cada caso foram revistas por dois patologistas. A técnica de hibridização *in situ* por fluorescência (FISH) para detetar a presença de rearranjos do *EWSR1* foi executada em lâminas com cortes colados de todos os casos obtidos a partir de blocos com tecidos impregnados em parafina.

Todos os casos de CEFTE revelaram rearranjo *EWSR1-FLI1* tanto no componente de pequenas células como no componente PTC. Dos 54 casos de PTC, 28 (51,9%) foram positivos, 20 (37,0%) foram negativos e 6 (11,1%) foram inconclusivos para o rearranjo *EWSR1*; em 2 casos PTC positivos foi detetado o rearranjo *EWSR1-FLI1*. Os casos de PTC clássico revelaram com maior frequência o rearranjo do *EWSR1* do que outras variantes de PTC (p=0.031). PTCs com rearranjo *EWSR1* revelaram menor frequência de núcleos com polissomia do *EWSR1* do que aqueles sem rearranjo do *EWSR1* (p=0.001). Dos 10 casos de SCN, 7 (70,0%) foram negativos e 3 (30,0%) inconclusivos para o rearranjo do *EWSR1*. Núcleos monossómicos foram mais frequentes (média de 44,3%) nos SCNs do que nos PTCs (p<0.001).

A presença do rearranjo *EWSR1-FLI1* na componente PTC de todos os CEFTEs estudados e a existência de rearranjo do *EWSR1* em alguns PTCs favorece a origem do CEFTE no PTC. A elevada frequência de rearranjos do EWSR1 em PTC pode representar um novo marcador de diagnóstico para estes tumores.

Palavras-chave: Carcinoma papilar da tiroide, Rearranjo *EWSR1*, Carcinoma de células pequenas, *EWSR1-FLI1*, Sarcoma de Ewing.

1 - INTRODUCTION

1.1 - THYROID GENERAL ASPECTS

The thyroid gland consists of two bulky lobes connected by a relatively thin isthmus, usually located below and anterior to the larynx (Figure 1). This organ develops embryologically from an evagination of the developing pharyngeal epithelium that descends from the foramen cecum at the base of the tongue to its normal position in the anterior neck (1).

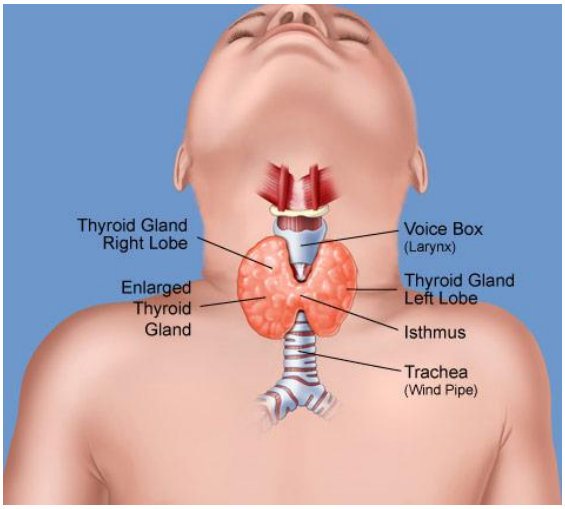

Figure 1 - Localization of thyroid gland. (www.med-health.net)

The thyroid is an endocrine organ that is responsible for the production and controlled release of thyroid hormones that are essential to fetal development and regulation of many metabolic processes (2). The thyroid parenchyma is constituted by lobules, each composed by 20 to 40 dispersed follicles, filled with thyroglobulin (TG), the iodinated precursor protein of active thyroid hormone.

The regulation of the thyroid metabolism is complex and involves the central nervous system. Briefly, the hypothalamus produce thyroid releasing hormone (TRH) that stimulates the pituitary gland for producing thyroid stimulating hormone (TSH). TSH binds to the receptor on the thyroid follicular epithelium cells which active these cells to convert TG into thyroxine (T_4) and lesser amounts of triiodothyronine (T_3) (Figure 2). T_4 and T_3 are released into the systemic circulation, where most of these peptides are reversibly bound to circulating plasma proteins (1).

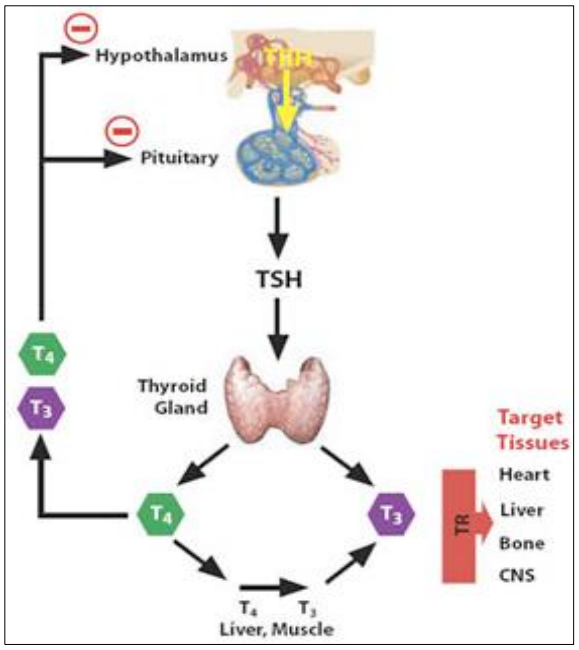

Figure 2 - Metabolism of thyroid gland. (www.myhousecallmd.com)

The lateral lobes of the thyroid also harbor the C-cells which are neuroendocrine cells responsible for the production of calcitonin. Both C-cells and squamoid main cells may constitute cellular aggregates, the so called solid cell nests (SCN) (2, 3).

The thyroid provides an attractive model to study the steps that are involved in the neoplastic process.

1.2 - PRIMARY SMALL CELL TUMOURS OF THE THYROID

Thyroid tumours can assume variable histological appearances, including the small cell phenotype that may occur in lymphoma, poorly differentiated carcinoma, medullary carcinoma, secondary neoplasms, primary extraeskeletal Ewing family tumours (PEEFTs) and other tumours with uncertain histogenesis (4-7).

The small cell tumours of the thyroid are rare and, for years, have been considered a major issue of debate (4). The differential diagnosis of small cell thyroid tumours is classically based upon the immunohistochemical profiles of these tumours (5) (Table 1).

Table 1 - Differential diagnosis of thyroid tumours with neuroendocrine differentiation.

	Expression of Neuroendocrine Markers		
Size of tumour cells	Calcitonin + CGRP + Chromogranin +	Calcitonin – CGRP + Chromogranin +	Calcitonin – CGRP – Chromogranin +
Large / intermediate cells	MTC Metastases of NET	C-cell derived calcitonin-free atypical MTC	Primary NET
Small cells	MTC Metastases of NET	Not described	Metastases of NET Paraganglioma Parathyroid tumour Primary NET Metastases of NET PEEFT/CEFTE (with neuroendocrine differentiation)

Legend: +, Positive; –, Negative; CGRP, Calcitonin gene related peptide; MTC, Medullary thyroid carcinoma; NET, Neuroendocrine tumour. (Eloy C. *et al*. 2014)

1.2.1 - PRIMARY EXTRAESKELETAL EWING FAMILY TUMOUR (PEEFT)

PEEFTs are rare bone or soft tissue sarcomas (8-10), commonly arising in the paravertebral region, chest wall, retroperitoneum, and lower extremities (11, 12). These neoplasms may also arise in other organs and sites, such as lung, skin, intestine, kidney and thyroid. PEEFTs are rare in the region of head and neck (13) and are usually diagnosed in childhood and young adults (9, 12, 14). In these cases the differential diagnosis from other sarcomas, carcinomas or lymphomas can be difficult (15). Histologically, PEEFT are characterized by a small round blue cell population (12, 13, 16) disclosing neuroectodermal differentiation at a variable extent (10, 12) (Figure 3).

PEEFT immunohistochemical profile includes a membrane diffuse expression for CD99 (Cluster of differentiation 99) (10, 14), however CD99 expression is not specific to PEEFT, as it has also been detected in other neoplasms (12). PEEFT has also expression for vimentin, synaptophysin and neuron specific enolase (NSE) and has absence of expression of cytokeratins and E-cadherin (5).

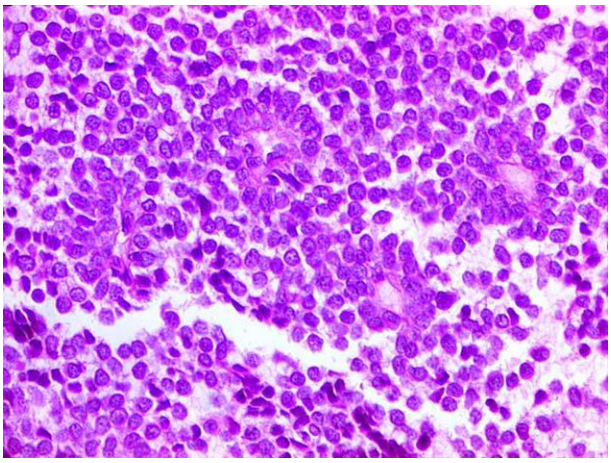

Figure 3 - Histological aspect of PEEFT (H&E - 200x). (Biswas G. *et al*. 2006)

PEEFTs are characterized by a typical chromosomal rearrangement involving the *EWSR1* gene (14, 17-19), that is thought to be the initiating event in the development of this neoplasm (14). The fusion products that result from *EWSR1* and partners rearrangement, commonly results from fusion of the N-terminal transcription-activating domain of *EWSR1* and the C-terminal DNA-binding domain of the fusion partner (9). One of the most common fusion partners of *EWSR1* is the *FLI1* gene (9, 20-22) (Figure 4). The *EWSR1-FLI1* rearrangement involving, respectively, the chromosome 22 and the chromosome 11, has been documented in 85% of the cases of soft tissues PEEFTs (8, 10, 16, 17) and in all of the cases of thyroid PEEFTs (5, 15, 22). *EWSR1-FLI1* codifies an oncoprotein and is recognized as a DNA-binding transcriptional regulator (21). Nearly 12 different in-frame *EWSR1-FLI1* chimeric transcripts have been observed containing different combinations of exons from *EWSR1* and *FLI1*, resulting in various combinations of *EWSR1* and *FLI1* genomic breakpoints (10, 12). These tumor-specific molecular rearrangements can be visualized by a “gold standard” method for fusion gene detection, fluorescence *in situ* hybridization (FISH) (9).

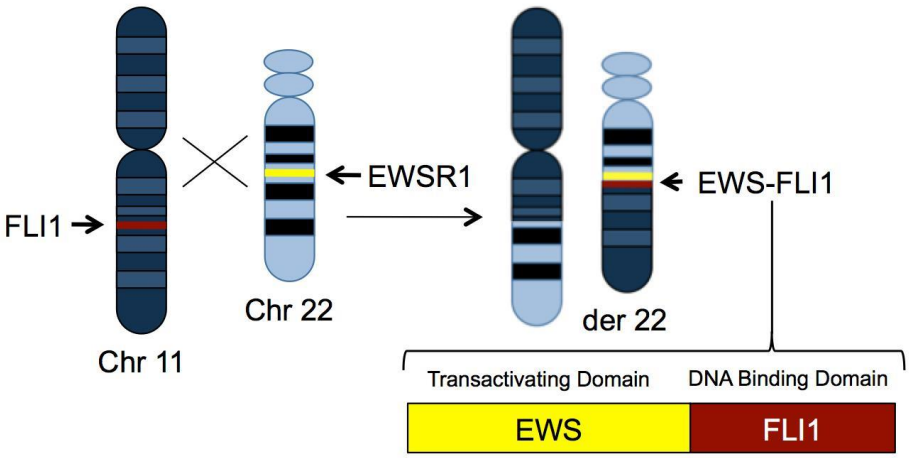

Figure 4 - Diagrammatic representation of the *EWSR1-FLI1* rearrangement (www.vai.org)

EWSR1-FLI1 rearrangement is useful for primary diagnosis of PEEFT, may provide prognostic information, and is considered an optimal drug marker for specific therapy (8, 19, 20).

1.2.2 - CARCINOMA OF THE THYROID WITH EWING FAMILY TUMOUR ELEMENTS (CEFTE)

The recent identification of a small cell carcinoma of the thyroid with Ewing family tumour elements (CEFTE) brought an extra difficulty to the small cell thyroid tumours differential diagnosis (4, 6). CEFTE are small cell thyroid tumours with epithelial differentiation that typically co-exist with papillary thyroid carcinoma (PTC) foci, and display vascular invasion (Figure 5), p63 and CD99 expression, negativity for TTF-1 and TG, Ewing sarcoma *EWSR1-FLI1* rearrangement and a favorable prognosis, despite their poorly differentiated appearance, high mitotic index and foci of necrosis (5-7). These CEFTE must be distinguished from the rare true PEEFTs arising in thyroid (15). Two hypotheses for the origin of CEFTE have been advanced:

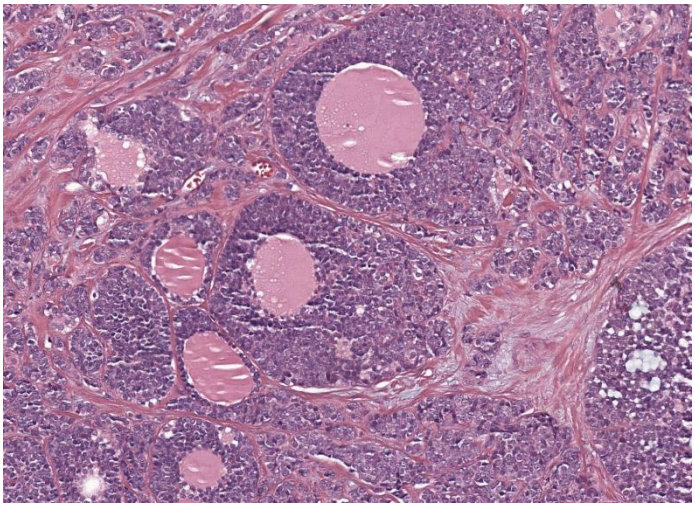

Figure 5 - Histological aspect of CEFTE (H&E - 100x)

- The topographic co-existence of a small cell tumour and PTC foci in CEFTE raised the hypothesis of a putative histogenic relation between these tumours, where PTC cells acquired the *EWSR1-FLI1* rearrangement and lost the thyroid differentiation (negative for TTF-1 and TG) (5, 7).
- The expression of p63 in the small cell component of CEFTE and the previous association between PTC and SCN that typically express p63, raised the possibility of an alternative histogenic hypothesis to CEFTE based upon an origin on SCNs (5, 7).

1.3 - PAPILLARY THYROID CARCINOMA (PTC)

Most cases of thyroid carcinoma occur in adults, although papillary thyroid carcinomas (PTC) (75% to 85% of cases) may also occur in childhood (23-26). PTC is the most frequent malignant thyroid neoplasm (27-30) and the most frequent type of endocrine cancer (24,

29, 31, 32). PTC is sporadic in about 95% of the cases and familial in the remaining 5% (29). The etiology of PTC is related to environmental, genetic and hormonal factors (33). Some of PTCs are associated with previous exposure to ionizing radiation (1, 24, 26). The usual presentation of PTC is a thyroid nodule or a neck mass (25) that can be present as solitary or multifocal lesions within the thyroid (34-36). According to recent epidemiological surveys the incidence rates of PTC (4 and 12 per 100.000 in men and women, respectively) are increasing (24). PTCs are indolent lesions with 10-year survival rates of 80% to 95% (25, 28, 30, 34).

PTC may co-exist with other thyroid tumours with small cell phenotype, namely poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) (5, 6, 31, 37, 38). PDTCs represent 4-7% of thyroid neoplasms (39). Morphologically, PDTCs are found in an intermediate position between differentiated and ATC, with differentiation loss and invasive growth, regional node and distant metastases (39, 40). PDTCs often occur in the female patients in sixth decade of live (39). ATCs represent less than 1% of all thyroid neoplasms and are among the most aggressive and lethal human neoplasms, with frequent distant metastases and poor prognosis (40). The mortality rate is higher than 90% in the first year, with a mean survival of 6 months after diagnosis (1, 37). Individuals with ATC are older than those with other types of thyroid cancer, with a mean age of 65 years at diagnosis (1, 38, 40).

It is usually accepted that PDTC and ATC occur either *de novo* or progress from a pre-existing well differentiated carcinoma (usually PTC) through a multistep process of genetic and epigenetic changes, typical of PTCs (for example *BRAF* mutation), that lead to clonal expansion and neoplastic development (25, 40).

PTCs, particularly microcarcinomas (found incidentally and measuring ≤10mm in diameter (33, 41) and PTCs with a conventional growth pattern, exhibit *BRAF* point mutations in up to 83% of cases, almost always in the same hot spot than melanoma (V600E) (24, 32, 38, 42-44). *BRAF* mutations have been associated to advanced age at time of diagnosis, male gender, absence of total encapsulation, extra-thyroid extension, lymph node metastases, distant metastases, recurrence and higher tumour stages (32). This mutation consists in a transversion from thymine to adenine in position 1799, leading to the substitution of a valine by a glutamate in codon 600 (24, 42, 45).

Other well known rearrangements have been documented in PTC, such as those involving the tyrosine kinase receptor gene *RET* (located on chromosome 10q11) (45-47), also known as *RET-PTC* rearrangement, is the most common rearrangement identified to date in thyroid papillary carcinomas (29, 41, 46, 48), and the *NTRK1* gene (neurotrophic tyrosine kinase receptor 1, located on chromosome 1q) (24), in 35% and 15% of cases, respectively (28, 42, 45, 48). *RET* and *NTRK1*, encode membrane tyrosine kinase (TK) receptors for

neural growth factors (49). Some studies have shown that the tumourigenic properties of *RET-PTC* and *NTRK1* result from the aberrant and persistent activation of their TK domain (24).

RET-PTC is more frequent in PTCs that have a classic architecture, microcarcinomas (31), PTCs associated with radiation exposure, tumours in children and young adults (24, 48), but are rarely found in familial cases (29). *RET-PTC* was the first rearrangement proposed as a marker for PTC, however, the initial enthusiasm was tempered by finding of this rearrangement in benign lesions like Hashimoto's thyroiditis (HT) and follicular adenomas and carcinomas of oncocytic type (50).

NTRK1 rearrangements are associated with radiation exposure and spontaneous thyroid tumours (49). The breakpoints of *NTRK1* rearrangements have been described in the perimembrane region upstream of TK domain of the receptor protein (33). These molecular alterations are an opportunity for develop news target therapies.

Despite all these molecular data, PTC diagnosis still depends on the identification of typical nuclei with large, irregular, clear, grooved aspect, occasionally displaying pseudo-inclusions, psammoma bodies and squamous metaplasia (24, 33, 36, 42) (Figure 6). PTCs can be diagnosed pre-operatively by fine needle aspiration biopsy (FNAB) and cytological evaluation. FNAB is helpful for distinguishing benign from malignant nodules and has an accuracy of 95% and false-negative rate of 4% to 6% (30).

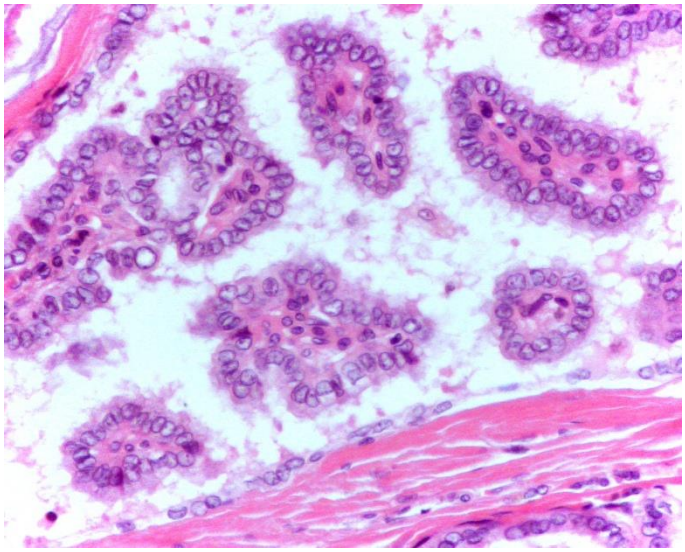

Figure 6 - Histological aspect of classic PTC (H&E - 200x)

PTCs may be solid or cystic, may have an infiltrative or expansive growth pattern, and may show direct extension beyond the thyroid to adjacent tissues (33). Lesional calcification is a common feature (26). Metastases occur frequently to adjacent cervical lymph nodes, which can be the initial manifestation of the disease (25, 26, 35), nevertheless, PTCs generally harbor a good prognosis (1, 26, 46). Despite the good prognosis, PTCs may give rise to recurrences and/or distant metastases, sometimes many years after diagnosis (32). Distant metastases of PTC to lungs and bones occur in 5-7% of cases (26, 45). In general, the prognosis is less favorable among elderly persons, large tumour size, male gender, multicentricity and in patients with invasion of extra-thyroidal tissues and/or distant metastases (1, 26, 32).

The first-line treatment of PTC is surgical excision (total or partial thyroidectomy), adjuvant radioiodine (^{131}I) ablation and TSH suppressive therapy (23, 30, 45). Usually the patients respond very well to these treatments but sometimes are resistant to radioactive iodine (RAI) (24, 45), and 20% of all PTC patients ultimately show disease progression (23). Recent study demonstrated that patients with progressive thyroid cancer, refractory to RAI, have responded to "targeted" multikinase inhibitors (for example selumetinib in combination with other agents), which inhibit angiogenesis and not the tumour cell, in PTCs that harbor the *BRAF* mutation (51). Sorafenib, also has been approved for differentiated thyroid cancer refractory to RAI with relevant secondary effects, justifying the need to explore new treatment approaches (45).

1.4 - SOLID CELL NESTS (SCN)

Solid cell nests of thyroid represent remnants of ultimobranchial body (52, 53) and are a normal component of the human thyroid gland (44, 53), found incidentally in about 60% of adult thyroids and in 89% of neonatal thyroids, if the entire glands were examined (44). SNC are irregular structures of about 1 mm in maximal diameter, which usually is located mainly in the middle or occasionally in the upper third of the thyroid lateral lobes, between the thyroid follicles (18). SCN display single or multiple clusters of 2 cell types referred to as main cells and C-cells (44, 52, 53). SCN may be difficult to distinguish from squamous metaplasia, primary or metastatic squamous cell carcinoma, papillary and medullary microcarcinoma and C-cell hyperplasia (52, 53).

The nuclei of SCN, centrally located, are round to oval and fusiform in shape with an eosinophilic cytoplasm (18, 44, 53) (Figure 7). SCN are predominently composed by non-keratinizing squamoid cells in solid nests or cystic formation filled with PAS-positive granular material after diastase digestion and acid mucins (18, 52, 53). This squamoid main cells are immunochemically positive for p63, galectin-3, carcinoembryonic antigen (CEA) and cytokeratins (CAM5.2, AE1/AE3, 34βE12, CK7 and CK19) (44, 52). Main cells of SCN display a basal/stem cell phenotype (p63 and basal cytokeratin positivity) (52, 53).

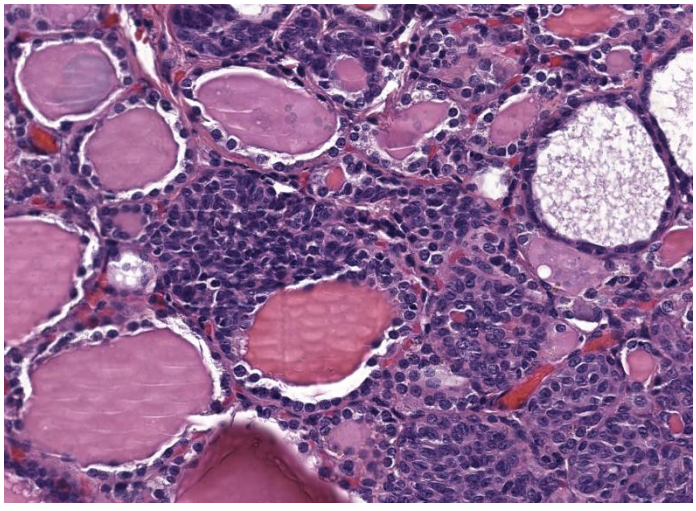

Figure 7 - Histological aspect of SCN (H&E - 200x)

2 - AIM OF THE STUDY

2 - AIM OF THE STUDY

In an attempt to understand the histogenesis of CEFTE, we searched for the *EWSR1-FLI1* rearrangements in the PTC component of CEFTEs, and studied the frequency of this rearrangement in a series of PTCs and in a series of SCNs.

3 - MATERIALS AND METHODS

3.1 - BIOLOGICAL SAMPLES

Thyroid lesions were retrieved from the Pathology Archive of Ipatimup Diagnostics at Ipatimup. A total of 3 CEFTEs (received in consultation), 54 consecutive PTCs non associated to small cell tumours and 10 SCNs topographically non associated to other lesions were collected from March 2011 to February 2015. The clinical data was collected from the patient files and the morphological features of each case were reviewed by two pathologist (CE and AP). For each case, all available haematoxylin and eosin stained sections were observed and one area (in PTCs and SCNs) or two (in CEFTE, comprehending the small cell component and the PTC component) were selected for fluorescence *in situ* hybridization analysis, in a single slide *per* case.

3.2 - FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

Fluorescence *in situ* hybridization (FISH) is the "gold standard" method used for fusion gene detection (23). FISH is a powerful technique for quantitative analysis of chromosomes and genes and can be applied in various samples, does not require cell culture or metaphase nuclei (54). FISH technique was performed in 4µm tissue sections obtained from paraffin embedded tissue of the 3 CEFTEs, 54 cases of PTCs and 10 SCNs. Tissue sections were extended on polysine adhesion slides (Thermo Scientific, Waltham, MA USA), following the next procedure:

- Desparaffinization with xylene and a decreasing alcohol series, until water type I.
- Antigen retrieval was performed with citrate buffer pH6.0 at 95ºC (Thermo Scientific, Waltham, MA USA).
    - ✓ It consists in breaking the methylenic bridges induced by formalin, allowing access to the present DNA in the nucleus and consecutive connection of the probe to the interest gene.
- Enzyme digestion with 0.5% pepsin at 37ºC (Sigma-Aldrich Quimica, Sintra, Portugal).
    - ✓ Pepsin is always prepared extemporaneously and to make active pepsin is used 1M hydrochloric acid (HCl), since it only reacts in acidic.
    - ✓ This step helps to recover the effects of covalent cross-links which were formed by the fixation in formalin.
    - ✓ A controlled enzyme digestion can improve the penetration of the probe into the interest gene.
- Codenatured at 79ºC and hybridization at 37ºC were performed overnight with a ZytoLight SPEC *EWSR1-FLI1* TrickCheck Probe (ZytoVision, Bremerhaven, Germany) in a SPOT-Light CISH Hybridizer.

- Wash in 2xSSC at 72ºC, for removing the excess of probe that did not react with the interest gene.
    - ✓ The stringency of 2xSSC solution is important to the quality of washing, which may be altered by the concentration of salts, temperature and the time in the solution.
- Nuclei were counterstained with DAPI/Dura-Tect mounting solution (ZytoVision, Bremerhaven, Germany).
    - ✓ Due to DAPI high selectivity for DNA as well as high permeability, this counterstain allows a good contrast nuclear, and a cytoplasm with a small background.

Appropriated positive and negative controls were used in each set of slides. Fluorescence hybridization signals were analyzed and recorded with a Zeiss Z.01 fluorescence microscope (Carl Zeiss, Germany). In each case, 50 nuclei were scored for the presence of *EWSR1* rearrangement, *EWSR1* monosomy, *EWSR1* polysomy and *EWSR1-FLI1* fusion. *EWSR1* rearrangement was considered if a signal separation of the 5'-orange and the 3'-green of the *EWSR1* probe was present. If this signal separation was observed together with the fusion of the 5'-orange signal of *EWSR1* and the blue signal of *FLI1*, the case was considered positive for *EWSR1-FLI1* fusion.

For establishing the cut-off level above which a case should be considered positive for *EWSR1* rearrangement, a FISH analysis was performed on 10 normal thyroid samples obtained from the Pathology Archive of Ipatimup Diagnostics at Ipatimup. The average percentage of *EWSR1* rearrangements in normal nuclei was 0.8%. Following De Vries *et al*., a value of three times the average percentage was used for calculation of the cut-off value (55). In the presence of ≥2.4% nuclei with break-apart sign of the region of the *EWSR1* gene, the case was considered positive for *EWSR1* rearrangement. Cases with nuclei without signals in less than 50 nuclei were classified as inconclusive. A normal nucleus for this chromosomal region was accepted when exhibiting 2 signals fusion of *EWSR1* (5'-orange and 3'-green) and 2 blue signals of *FLI1* separated. Monosomy was considered when the number of signals in each nucleus was inferior to those observed the normal nuclei, and polysomy was considered when the number of signals in each nucleus was superior to those observed the normal nuclei.

FISH is a laborious and time-consuming technique that requires the observation of a large number of nuclei by an experienced operator. However, this method provides important information on the percentage of cells harboring the *EWSR1* rearrangement.

3.3 - STATISTIC ANALYSIS

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20 for Windows. The Pearson´s chi-square (χ^2) test (or Fisher exact probability test if appropriate) was used for comparison of qualitative variables and the t-test, Mann-Whitney test and Pearson´s correlation were used for quantitative variables. The level of significance was set at $p<0.05$.

4 - RESULTS

4.1 - CARCINOMA OF THE THYROID WITH EWING FAMILY TUMOUR ELEMENTS (CEFTE)

Three cases of CEFTE were included in the study, from 2 males and 1 female, with mean age of 32 years-old (24-42 years) and a mean tumour size of 33mm. All of the CEFTE cases disclosed expansive growth, vascular invasion and co-existence of the small cell component with a PTC component.

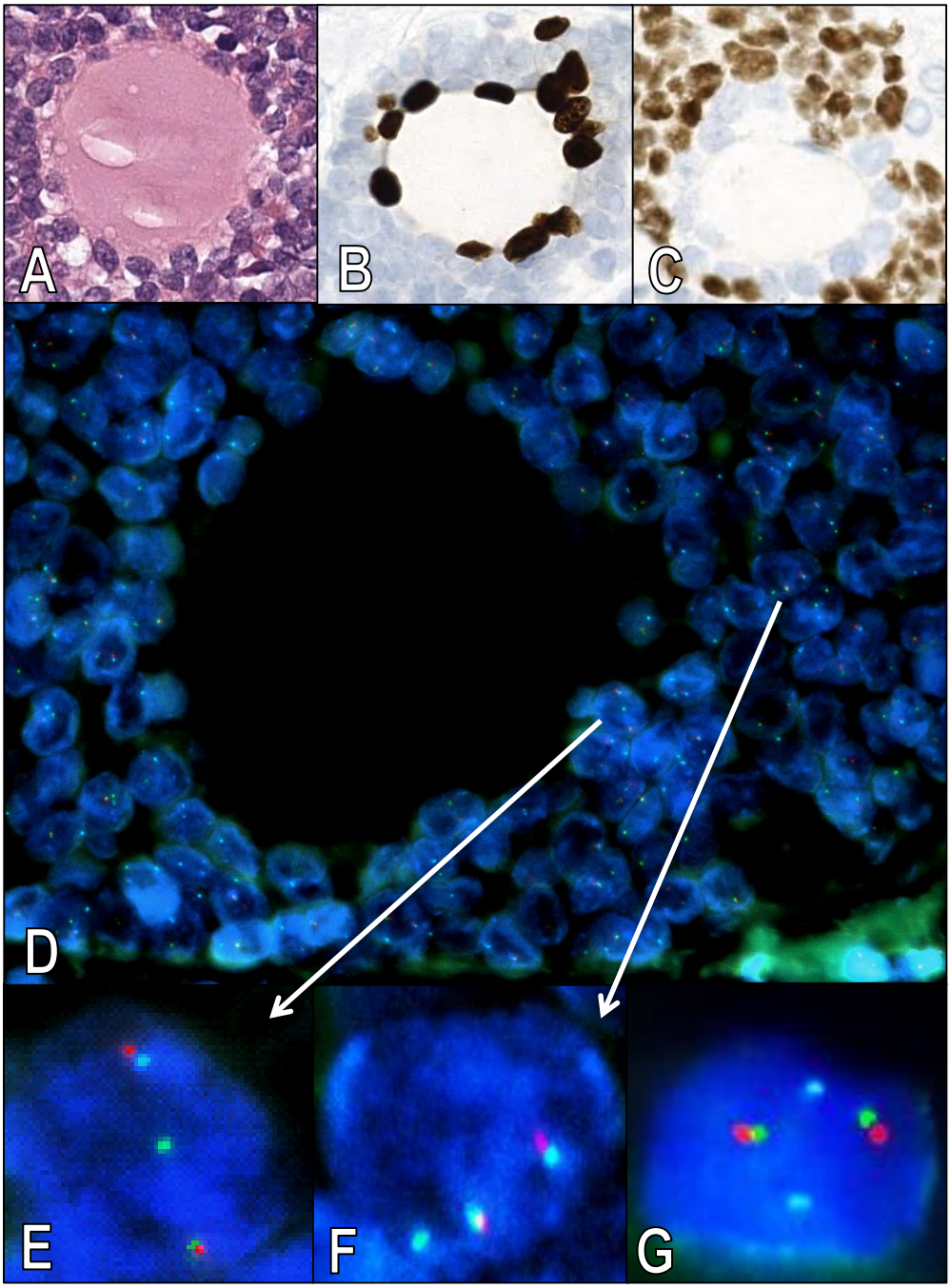

Figure 8 - CEFTE case disclosing a follicle of PTC component surrounded by small cells (A: H&E; 400x). The PTC component discloses TTF-1 expression at variance with the small cells (B: TTF-1; 400x) but does not express p63, that is expressed in the small cells (C: p63; 400x). The FISH analysis of this area demonstrates the presence of *EWSR1* break apart signals with fusion signals with *FLI1* in both the cells of the PTC and the small cells – one normal signal of *EWSR1* (5'-orange and 3'-green) and one fusion signal with FLI1 (5'-orange

signal of *EWSR1* fused with one blue signal of *FLI1*) (D,E,F: 600x). In G there is a normal nucleus of the normal appearing thyroid tissue for comparison purposes where it is possible to identify two signals of *EWSR1* (5'-orange and 3'-green) and two blue signals of *FLI1* separated (600x).

The small cell component of CEFTE was positive for p63 and CD99, as reported before (7). The PTC foci were positive for TTF-1 and negative for p63. The follow-up period for each case is 3, 4 and 15 years, and the patients are alive and well without signs of the disease. All of the three studied CEFTEs were positive for the *EWSR1-FLI1* rearrangement, both in small cell and PTC components (Figure 8).

4.2 - PAPILLARY THYROID CARCINOMA (PTC)

Table 2 summarizes the features of the 54 PTCs cases, including 43 females (79.6%) and 11 males (20.4%) with mean age of 49 years-old at the diagnosis (11-78 years) and mean tumour size of 17mm (5-53mm). This series included 11 classic PTCs (20.4%), 18 follicular variants of PTCs (33.3%), 19 papillary microcarcinomas (35.2%) and 6 trabecular/solid variants of PTCs (11.1%). PTCs disclosed infiltrative growth pattern in 22 cases (40.7%) and expansive growth patterns in 32 cases (59.3%). 24 cases (44.4%) were multicentric and 30 cases (55.6%) were unifocal. It was observed vascular invasion in 19 cases (35.2%) and in 35 cases (64.8%) not. 10 cases (18.5%) had extra-thyroid extension while 44 cases (81.5%) were limited to the thyroid parenchyma. Only 7 cases (13.0%) developed lymph nodes metastases and none of them had distant metastasis. The other thyroid was normal in 33 cases (61.1%), had Hashimoto's thyroiditis in 12 cases (22.2%), thyroiditis in 4 cases (7.4%) and goiter in 5 cases (9.3%). The tumour was in the right lobe in 28 cases (51.9%), in the left lobe in 23 cases (42.5%) and in the isthmus in 3 cases (5.6%). Capsule was present in 28 cases (51.9%) and absence in 26 cases (48.1%). The majority of the cases were in stage I (42 cases; 77.8%).

Table 2 - Clinico-pathological features of the 54 PTC cases

Case ID	Gender	Age (year)	Size (mm)	Variant	GP	Mult	VI	ExE	LNM	OT	Loc	Cap	St
A1	F	32	9	Micro	Inf	–	–	+	–	Nm	RL	–	I
A2	F	78	15	Classic	Exp	+	–	–	–	HT	Is	–	I
A3	F	52	8	Micro	Inf	–	–	–	–	Nm	LL	–	I
A4	F	37	22	Follicular	Exp	–	–	–	–	G	RL	+	I
A5	F	11	16	Trab/Solid	Inf	+	–	–	–	HT	LL	–	I
A6	F	76	10	Follicular	Exp	–	–	–	–	G	LL	–	I
A7	M	39	29	Classic	Exp	+	+	+	+	Nm	RL	+	I
A8	F	38	15	Classic	Inf	–	–	–	–	Nm	RL	–	I
A9	F	45	15	Classic	Exp	+	–	–	–	Nm	LL	+	I
A10	F	29	9	Micro	Inf	+	+	+	+	HT	RL	–	I
A11	F	46	17	Follicular	Exp	–	+	–	–	Nm	RL	+	I

A12	F	62	8	Micro	Inf	+	–	–	–	HT	LL	–	I
A13	F	73	10	Micro	Exp	–	+	–	–	Nm	LL	+	I
A14	F	78	8	Micro	Exp	+	+	+	–	Nm	LL	+	III
A15	F	46	18	Follicular	Exp	–	–	–	–	HT	LL	+	I
A16	M	44	12	Classic	Exp	+	+	–	+	Nm	LL	+	I
A17	M	56	45	Follicular	Exp	–	–	–	–	Nm	LL	+	III
A18	M	63	20	Follicular	Exp	+	–	–	–	T	Is	+	I
A19	F	52	35	Follicular	Exp	+	–	–	–	HT	RL	+	II
A20	F	27	10	Micro	Inf	–	+	–	+	Nm	RL	–	I
A21	F	42	26	Classic	Inf	+	+	+	+	HT	Is	–	I
A22	F	54	35	Follicular	Exp	–	–	–	–	Nm	RL	+	II
A23	F	58	7	Micro	Inf	+	+	+	–	Nm	LL	–	III
A24	F	60	14	Trab/Solid	Exp	–	–	–	–	Nm	LL	+	I
A25	F	20	23	Follicular	Exp	–	+	–	–	Nm	LL	+	I
A26	F	36	20	Follicular	Exp	+	–	–	–	HT	LL	+	I
A27	F	45	14	Follicular	Exp	–	–	–	–	Nm	RL	–	I
A28	M	35	6	Micro	Exp	+	–	–	–	Nm	LL	+	I
A29	F	39	17	Classic	Inf	+	+	–	+	Nm	RL	–	I
A30	F	18	28	Follicular	Exp	–	–	–	–	Nm	RL	+	I
A31	F	54	14	Classic	Exp	+	–	–	–	Nm	RL	+	I
A32	M	34	50	Follicular	Exp	+	–	–	–	G	RL	+	I
A33	M	35	53	Follicular	Exp	+	–	–	–	Nm	LL	+	I
A34	M	55	11	Classic	Inf	–	–	–	–	G	RL	–	I
A35	F	20	13	Trab/Solid	Exp	–	–	–	–	T	RL	–	I
A36	F	54	6	Micro	Exp	–	–	–	–	HT	LL	+	I
A37	F	57	6	Micro	Inf	–	–	–	–	G	RL	–	I
A38	F	39	8	Micro	Inf	–	–	–	–	Nm	RL	–	I
A39	F	48	6	Micro	Exp	+	–	–	–	HT	RL	–	I
A40	F	48	7	Micro	Inf	–	+	+	–	Nm	RL	–	III
A41	F	47	15	Trab/Solid	Exp	–	+	–	–	Nm	LL	+	I
A42	M	47	17	Classic	Inf	+	+	–	–	Nm	RL	–	I
A43	F	40	5	Micro	Inf	–	–	–	–	T	LL	–	I
A44	F	78	9	Micro	Inf	+	–	–	–	Nm	LL	–	I
A45	F	57	18	Follicular	Exp	–	+	–	–	HT	RL	+	I
A46	F	66	34	Follicular	Exp	+	–	–	–	Nm	RL	+	II
A47	F	40	6	Micro	Inf	–	–	+	–	Nm	RL	–	I
A48	M	68	46	Follicular	Exp	–	+	–	–	Nm	RL	+	III
A49	F	49	15	Classic	Inf	+	+	–	+	HT	RL	–	III
A50	F	53	14	Follicular	Exp	–	–	–	–	Nm	RL	+	I
A51	F	71	21	Trab/Solid	Exp	–	–	–	–	Nm	RL	+	II
A52	M	63	22	Trab/Solid	Inf	+	+	+	–	Nm	LL	–	III
A53	F	54	10	Micro	Inf	–	–	–	–	T	LL	–	I
A54	F	54	9	Micro	Inf	–	+	+	–	Nm	LL	–	III

Legend: M, male; F, female; Micro, Microcarcinoma; Trab/Solid, Trabecular/Solid; GP, Growth pattern; Inf, Infiltrative; Exp, Expansive; Mult, Multicentricity; VI, Vascular invasion; ExE, Extra-thyroid extension; LNM, Lymph node metastases; *EWSR1* rearr, *EWSR1* rearrangement; +, Present; –, Absent; OT, Other Thyroid; Nm, Normal; HT, Hashimoto's thyroiditis; G, Goiter; T, Thyroiditis; Loc, Localization of tumour; RL, Right lobe; LL; Left lobe; Is, Isthmus; Cap, Capsule; St, Stage.

Considering the occurrence of *EWSR1* rearrangement, a total of 28 cases (51.9%) were positive, 20 cases (37.0%) were negative and 6 cases (11.1%) were inconclusive. Two cases (A21 and A32) disclosed the *EWSR1-FLI1* fusion signal (7.1% in 28 positive cases, 3.7% in a total of 54 cases) (Figure 9). The percentage of polysomic nuclei in PTC case ranged from 0 to 40% (mean value=13.3%). The percentage of monosomic nuclei in PTC case ranged from 2 to 44% (mean value=17.2%) (Table 3).

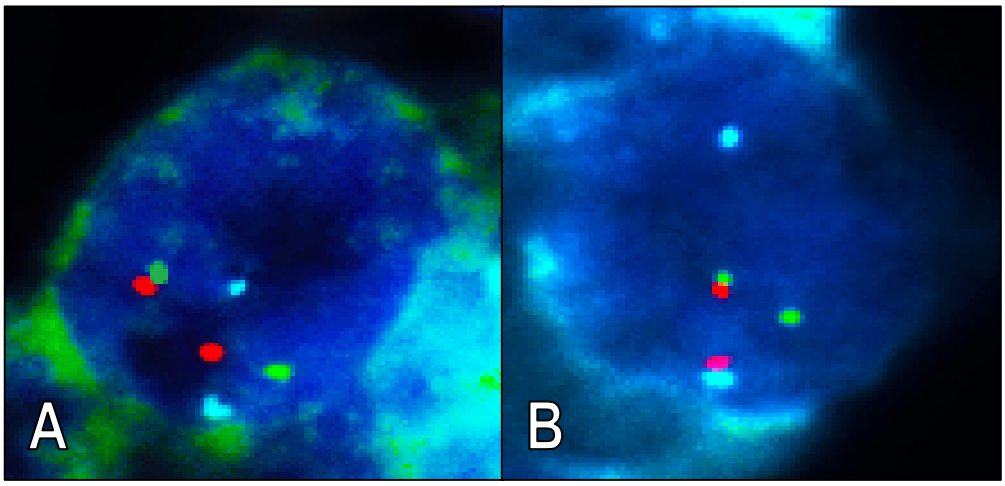

Figure 9 - The FISH analysis demonstrates the presence of *EWSR1* break apart signals without fusion signals with *FLI1* – one signal fusion *EWSR1* (5'orange and 3'green), two separated 3'green and 5'orange signals of *EWSR1* and two separated blue signals of *FLI1* (A:600x), and demonstrates the presence of *EWSR1* break apart signal with fusion signal with *FLI1* – one normal signal of *EWSR1* (5'-orange and 3'-green) and one fusion signal with *FLI1* (5'-orange signal of *EWSR1* fused with one blue signal of *FLI1*) (B:600x).

Table 3 - FISH analysis of the 54 PTC cases

Case ID	*EWSR1* rearrangement	*EWSR1* polysomy (% cells)	*EWSR1* monosomy (% cells)
A1	I	I	I
A2	P	4	6
A3	I	I	I
A4	P	20	8
A5	I	I	I
A6	N	14	18
A7	P	4	28
A8	N	40	10
A9	P	16	8
A10	N	20	12
A11	P	0	36
A12	N	14	32
A13	N	24	18
A14	N	36	10
A15	P	2	44
A16	P	10	8
A17	N	24	4

A18	N	20	18
A19	P	18	10
A20	N	18	16
A21	P	12	6
A22	N	8	42
A23	N	22	6
A24	P	14	16
A25	N	16	32
A26	N	8	18
A27	N	34	8
A28	P	6	20
A29	P	12	14
A30	N	2	42
A31	I	I	I
A32	P	4	8
A33	P	0	26
A34	P	4	18
A35	I	I	I
A36	P	6	8
A37	N	14	34
A38	P	2	16
A39	P	14	12
A40	N	28	8
A41	P	0	34
A42	P	12	22
A43	P	12	8
A44	P	12	14
A45	P	12	10
A46	P	10	16
A47	P	14	12
A48	N	4	22
A49	P	10	2
A50	N	6	40
A51	N	34	6
A52	I	I	I
A53	P	6	12
A54	P	18	8

Legend: P, Positive; N, Negative; I, Inconclusive.

Classic PTC disclosed more often the *EWSR1* rearrangement than the other PTC variants (90.0% in classic PTCs and 50.0% in other variants; p=0.031). The PTC cases with *EWSR1* rearrangement had lower percentage of nuclei with polysomy than PTC cases without *EWSR1* rearrangement (mean of 9.1% vs 19.3%, respectively; p=0.001). There was no statistically difference of the percentage of nuclei with monosomy between the PTC cases with or without *EWSR1* rearrangement (mean of 15.4% vs 19.8%, respectively; p=0.185).

There was no statistically significant association between PTC clinico-pathological features and the FISH results (Table 4).

Table 4 - Statistic analysis of clinico-pathological features and results of the FISH analysis in PTCs

	***EWSR1* with rearrangement**	***EWSR1* without rearrangement**	***p***
Gender (Female / Male)	21 / 7	17 / 3	0.488 [a]
Age (Mean ± Standard Deviation)	48.6 ± 11.6	51.5 ± 18.4	0.535 [b]
Size (Mean ± Standard Deviation)	17.8 ± 12.5	17.8 ± 12.2	0.997 [b]
Variant (Classic / Other)	9 / 19	1 / 19	**0.031** [a]
Growth pattern (Infiltrative / Expansive)	11 / 17	7 / 13	0.762 [c]
Multicentricity (Present / Absent)	15 / 13	6 / 14	0.105 [c]
Vascular invasion (Present / Absent)	10 / 18	8 / 12	0.762 [c]
Extra-thyroid extension (Present / Absent)	4 / 24	4 / 16	0.703 [a]
Lymph node metastases (Present / Absent)	5 / 23	2 / 18	0.683 [a]
Other thyroid (Normal / Other)	15 / 13	14 / 6	0.251 [c]
Localization of tumour (LD / LE)	14 / 12	11 / 8	0.787 [c]
Capsule (Present / Absent)	15 / 13	11 / 9	0.922 [c]
Capsule invasion (Present / Absent)	11 / 4	5 / 6	0.228 [a]
Stage (I / Other)	24 / 4	13 / 7	0.162 [a]
***EWSR1* polysomy** (Mean ± Standard Deviation)	9.1 ± 5.9	19.3 ± 11.1	**0.001** [b]
***EWSR1* monosomy** (Mean ± Standard Deviation)	15.4 ± 10.1	19.8 ± 12.8	0.185 [b]

Legend: a, Fisher exact probability test; b, t-test; c, Pearson's chi-square (x^2)

4.3 - SOLID CELL NESTS (SCN)

Table 5 summarizes the clinico-pathological features and the result of the FISH analysis in the SCNs series. Ten SCNs were detected in thyroids from 7 females and 3 males, with a mean age of 54 years-old (32-74 years). Seven SCNs were negative for *EWSR1* rearrangement (70.0%) and 3 cases were inconclusive (30.0%). All studied SCNs with conclusive results disclosed *EWSR1* monosomy in at least 30% of the tumour cells (mean of 44.3%) while only 18.8% of PTCs disclosed this percentage of *EWSR1* gene monosomy (mean of 17.2%) ($p<0.001$) (Figure 10). None of the studied SCNs disclosed *EWSR1* rearrangements (with or without the *FLI1* gene) or *EWSR1* polysomy.

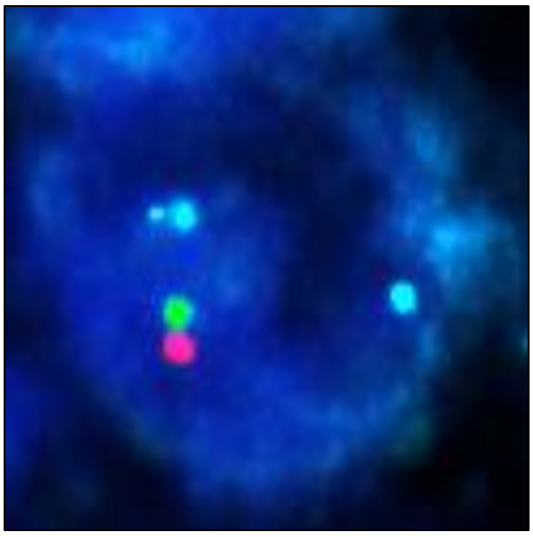

Figure 10 - The FISH analysis demonstrates the presence of *EWSR1* monosomy – one normal signal of *EWSR1* (5'-orange and 3'-green), and two separated blue signals of *FLI1* (A: 600x).

Table 5 - Clinico-pathological features and results of the FISH analysis of SCN cases

Case ID	Gender	Age	*EWSR1* polysomy (% cells)	*EWSR1* monosomy (% cells)	EWSR1 rearrangement
A55	M	70	0	60	N
A56	F	47	I	I	I
A57	F	46	I	I	I
A58	M	63	I	I	I
A59	F	32	2	32	N
A60	F	56	0	44	N
A61	M	41	0	42	N
A62	F	74	0	30	N
A63	F	60	0	48	N
A64	F	NA	0	54	N

Legend: M, male; F, female; NA, Not available; N, Negative; I, Inconclusive

5 - DISCUSSION

5 - DISCUSSION

CEFTEs are exceptionally rare small cell tumours of the thyroid with apparently favorable prognosis but are difficult to distinguish from the usually aggressive PEEFTs, as they both have the *EWSR1-FLI1* rearrangement. At variance with PEEFTs, CEFTE disclose a PTC component that shares with the small cell component of these tumours the *EWSR1-FLI1* rearrangement. Reported PEEFTs do not exhibit a PTC component even if the tumour develops in the thyroid (22). Besides the putative histogenetic link between CEFTE and PTCs, the SCNs were the alternative hypothesis for the origin of CEFTE, as previously described (5, 7). SCNs represent remnants of ultimobranchial body, are a normal component of the human thyroid gland and are positive for p63 (44, 52) as it happens in CEFTE (44, 53). In our series, none the studied SCNs disclosed the *EWSR1* rearrangement, a result that does not favour the hypothesis of CEFTEs having origin in SCNs; an assumption limited by the small number of samples that must be confirmed in a larger series of SCNs.

The histogenetic relationship between SCN and PTC previously proposed (44) is also debatable at this point, considering that SCNs did not disclose *EWSR1* polysomic nuclei and had a higher percentage of monosomic nuclei than PTC cases (0% vs 13.3% and 44.3 vs 17.2%, respectively).

The presence of the *EWSR1-FLI1* rearrangement in the PTC component of all the studied CEFTEs of our series strongly suggests a histogenetic relation between CEFTE and PTC and reinforces the individualization of CEFTE as a single clinico-pathological entity. Taking into consideration that *EWSR1* rearrangement occurs in 52% of PTC cases (without small cell component), the assumption that CEFTE may arise from a previously formed PTC that acquired the *EWSR1-FLI1* fusion, becomes the more probable interpretation for CEFTE histogenesis.

The *EWSR1* rearrangement detected in non small cell associated-PTCs is not occurring, in most of the cases, with the *FLI1* gene. Probably, when the fusion of the *FLI1* with the *EWSR1* occurs in a cell with other background genetic / epigenetic alterations there is loss of thyroid differentiation and acquisition of small cell phenotype, such as may happen in CEFTE. The two cases that disclosed *EWSR1-FLI1* gene rearrangement, one follicular variant PTC and one classic PTC, did not present any particular clinico-pathological features to be individualized as indicating a special phenotype. The role of *EWSR1-FLI1* gene rearrangement in PTC neoplastic development should be studied in a larger series of positive cases with an extended follow-up to evaluate any association with prognosis and with transformation and subsequent overgrowth by CEFTE. Remains to be explained the mechanism by which the tumour cells lose their follicular differentiation, namely the

expression of TTF-1 and thyroglobulin, and acquire diffuse p63 expression. Poorly differentiated carcinoma and anaplastic carcinoma are examples of follicular derived tumours that may lose thyroglobulin expression, and anaplastic carcinoma may also disclose TTF-1 absence of expression. Nevertheless, both poorly differentiated and anaplastic carcinomas result from neoplastic progression of PTC in elderly patients and are associated to poor prognosis at variance with CEFTE that occurs in young patients and appears to have a favourable prognosis. The mechanism that underlays the acquisition of p63 expression can probably be similar to those mechanisms occurring in squamous metaplasia of follicular tumour cells, mucoepidermoid carcinoma, SETTLE (spindle cell tumour with thymus like elements) and CASTLE (carcinoma with thymus like elements) that also express p63. The abovementioned alterations in the differentiation of PTC follicular cells that appear to be related to progression into CEFTE should be distinct from those occurring in PEEFT of the thyroid that do not express p63 and cytokeratins as in CEFTE. PEEFT of the thyroid follow a clinical course similar to PEEFT in other locations and probably arise from neuroectodermal cells such as those that arise in soft tissues and bone. This is the first report of the presence of *EWSR1* rearrangement in PTC. The results of our study indicate that *EWSR1* rearrangement is present in about half of the cases, with a higher frequency in classic PTC than in PTC variants. Previous studies highlighting that *BRAF* mutation was the most frequent genetic alteration in classic PTC (32) are now debatable, as the *EWSR1* rearrangement is detected in 90.0% of classic PTC of our series.

PTC is a carcinoma rich in rearrangements such a *RET-PTC*, *PAX8-PPARgama*, *NTRK1* rearrangements, *BRAF* rearrangements and the new *ALK* rearrangements (56). The *EWSR1* rearrangement is more frequent in PTC than rearrangements involving *RET* and *NTRK1* genes, that also have a higher frequency in classic PTC than in PTC variants (42, 48). The 26 PTC cases with *EWSR1* rearrangement of our series, in which the fusion with the *FLI1* gene was not observed, need to be further studied for the identification of the partner gene.

In our series, CEFTE cases have had so far a favorable prognosis and none of the PTC cases disclosed distant metastases that may preclude a guarded prognosis. Taking this in consideration, we observed that the presence of *EWSR1* rearrangement, polysomy or monosomy was not associated with clinico-pathological features indicative of clinical aggressiveness in PTCs. The high frequency of *EWSR1* rearrangement in our series of PTC with low aggressiveness may indicate that this rearrangement is, in fact, not associated to prognosis as it happens with *ALK* rearrangement (57) and at variance with *RET-PTC* rearrangement that associates with an indolent “bonsai” phenotype that do not tend to evolve into poorly differentiated or anaplastic carcinoma (29). Nevertheless, the existence of a specific therapy for Ewing sarcomas (20) that harbor the *EWSR1-FLI1* rearrangement

should be kept in the back of the mind as a possible therapy for patients with clinically aggressive PTC cases refractory to radioactive iodine treatment (45) that display the *EWSR1-FLI1* rearrangement.

Finally, there is a potential usefulness of the *EWSR1* rearrangement in the diagnosis of PTC, particularly in the setting of fine needle aspiration biopsy (FNAB). Unfortunately, the rearrangement is particularly prevalent in classic PTCs which are the easiest to diagnose by FNAB.

6 - CONCLUSION AND FUTURE PERSPECTIVES

6.1 - CONCLUSION

Summing up, we studied a series of PTC and SCN cases based in previous studies that defended that CEFTEs had origin in one of these entities. None of the SCNs studied by us were positive for the *EWSR1* rearrangement with or without *FLI1* fusion. In the PTC cases, 52% of these cases were positive for the *EWSR1* rearrangement and two cases were positive for the *EWSR1-FLI1* rearrangement. This observation supports the origin of CEFTEs in PTCs.

This is the first study where *EWSR1-FLI1* rearrangement is described. This rearrangement occurs in a higher frequency in classic PTC than in other PTC variants.

In our series, *EWSR1* rearrangement was not associated with clinico-pathological features indicative of clinical aggressiveness in PTCs, which may indicate that this rearrangement is not associated to prognosis.

The existence of a specific therapy for Ewing sarcomas with *EWSR1-FLI1* rearrangement may be a possible therapy for patients with PTC that display *EWSR1-FLI1* rearrangement and are refractory to radioactive iodine treatment.

6.2 - FUTURE PERSPECTIVES

Study the *EWSR1* rearrangement in other thyroid tumours and benign lesions.

Study the role of *EWSR1* rearrangement in the cytological diagnosis of thyroid nodules.

Study a larger series of PTC cases to evaluate associations between prognosis and presence of *EWSR1* rearrangement.

Study the 26 PTC cases of our series with *EWSR1* rearrangement, where the fusion with the *FLI1* gene was not observed, for the identification of the partner gene.

7 - REFERENCES

7 - REFERENCES

1. Robbins SL, Cotran RS. Pathologic basis of disease: Saunders; 1979.
2. J R. Rosai and Ackerman's Surgical Pathology. London: Elsevier Mosby; 2011.
3. V L. Surgical Pathology of the thyroid – Major problems in pathology. Philadelphia Saunders; 1990.
4. Cruz J, Eloy C, Aragüés JM, Vinagre J, Sobrinho-Simões M. Small-cell (basaloid) thyroid carcinoma: a neoplasm with a solid cell nest histogenesis? International journal of surgical pathology. 2011:1066896911405320.
5. Eloy C, Cameselle-Teijeiro JM, Rousseau E, Sobrinho-Simões M. Small Cell Tumors of the Thyroid Gland A Review. International journal of surgical pathology. 2014;22(3):197-201.
6. Eloy C, Oliveira M, Vieira J, Teixeira MR, Cruz J, Sobrinho-Simões M. Carcinoma of the Thyroid With Ewing Family Tumor Elements and Favorable Prognosis: Report of a Second Case. International journal of surgical pathology. 2013:1066896913486696.
7. Eloy C, Cameselle-Teijeiro J, Vieira J, Teixeira MR, Cruz J, Sobrinho-Simões M. Carcinoma of the Thyroid With Ewing/PNET Family Tumor Elements A Tumor of Unknown Histogenesis. International journal of surgical pathology. 2014;22(6):579-81.
8. Jiang Y, Ludwig J, Janku F. Targeted therapies for advanced Ewing sarcoma family of tumors. Cancer treatment reviews. 2015;41(5):391-400.
9. Romeo S, Dei Tos AP. Soft tissue tumors associated with EWSR1 translocation. Virchows Archiv. 2010;456(2):219-34.
10. Rekhi B, Vogel U, Basak R, Desai SB, Jambhekar NA. Clinicopathological and Molecular Spectrum of Ewing Sarcomas/PNETs, Including Validation of EWSR1 Rearrangement by Conventional and Array FISH Technique in Certain Cases. Pathology & Oncology Research. 2014;20(3):503-16.
11. Adapa P, Chung T-W, Popek EJ, Hunter JV. Extraosseous Ewing sarcoma of the thyroid gland. Pediatric radiology. 2009;39(12):1365-8.
12. Ozaki T. Diagnosis and treatment of Ewing sarcoma of the bone: a review article. Journal of Orthopaedic Science. 2015;20(2):250-63.
13. Biswas G, Hingmire S, Bagwan I, Prasad N, Bakshi A, Chinoy R, et al. Primitive neuroectodermal tumor (PNET) of thyroid. Oral Oncology Extra. 2006;42(5):198-9.
14. Burchill SA. Molecular abnormalities in Ewing's sarcoma. 2008.

15. Maldi E, Monga G, Rossi D, Tosoni A, Mezzapelle R, Boldorini R. Extra-osseous Ewing sarcoma of the thyroid gland mimicking lymphoma recurrence: A case report. Pathology-Research and Practice. 2012;208(6):356-9.
16. Newby R, Rowe D, Paterson L, Farquharson MA, MacDuff E, Coupe A, et al. Cryptic EWSR1-FLI1 fusions in Ewing sarcoma: potential pitfalls in the diagnostic use of fluorescence in situ hybridization probes. Cancer genetics and cytogenetics. 2010;200(1):60-4.
17. Dragoescu E, Jackson-Cook C, Domson G, Massey D, Foster WC. Small cell osteosarcoma with Ewing sarcoma breakpoint region 1 gene rearrangement detected by interphase fluorescence in situ hybridization. Annals of diagnostic pathology. 2013;17(4):377-82.
18. Harach HR. Solid cell nests of the thyroid. The Journal of pathology. 1988;155(3):191-200.
19. de Alava E, Gerald WL. Molecular biology of the Ewing's sarcoma/primitive neuroectodermal tumor family. Journal of Clinical Oncology. 2000;18(1):204-.
20. Tancredi R, Zambelli A, DaPrada G, Fregoni V, Pavesi L, Riccardi A, et al. Targeting the EWS–FLI1 transcription factor in Ewing sarcoma. Cancer chemotherapy and pharmacology. 2015;75(6):1317-20.
21. Selvanathan SP, Graham GT, Erkizan HV, Dirksen U, Natarajan TG, Dakic A, et al. Oncogenic fusion protein EWS-FLI1 is a network hub that regulates alternative splicing. Proceedings of the National Academy of Sciences. 2015;112(11):E1307-E16.
22. Chan JM, Bilodeau E, Celin S, Nikiforov Y, Johnson JT. Ewing sarcoma of the thyroid: Report of 2 cases and review of the literature. Head & neck. 2013;35(11):E346-E50.
23. Zhang T, Lu Y, Ye Q, Zhang M, Zheng L, Yin X, et al. An evaluation and recommendation of the optimal methodologies to detect RET gene rearrangements in papillary thyroid carcinoma. Genes, Chromosomes and Cancer. 2015;54(3):168-76.
24. Trovisco V, Soares P, Preto A, Castro P, Máximo V, Sobrinho-Simões M. Molecular genetics of papillary thyroid carcinoma: great expectations. Arquivos Brasileiros de Endocrinologia & Metabologia. 2007;51(5):643-53.
25. Al-Qsous W, Miller ID. Anaplastic transformation in lung metastases of differentiated papillary thyroid carcinoma: an autopsy case report and review of the literature. Annals of diagnostic pathology. 2010;14(1):41-3.
26. LiVolsi VA. Papillary thyroid carcinoma: an update. Modern Pathology. 2011;24:S1-S9.

27. Girardi FM, Barra MB, Zettler CG. Papillary thyroid carcinoma: does the association with Hashimoto's thyroiditis affect the clinicopathological characteristics of the disease? Brazilian journal of otorhinolaryngology. 2015;81(3):283-7.
28. Soares P, Fonseca E, Wynford-Thomas D, Sobrinho-Simões M. Sporadic ret-rearranged papillary carcinoma of the thyroid: a subset of slow growing, less aggressive thyroid neoplasms? The Journal of pathology. 1998;185(1):71-8.
29. Soares P, Trovisco V, Rocha AS, Lima J, Castro P, Preto A, et al. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene. 2003;22(29):4578-80.
30. Caron NR, Clark OH. Papillary thyroid cancer. Current treatment options in oncology. 2006;7(4):309-19.
31. Santoro M, Melillo RM, Fusco A. RET/PTC activation in papillary thyroid carcinoma: European Journal of Endocrinology Prize Lecture. European Journal of Endocrinology. 2006;155(5):645-53.
32. Eloy C, Santos J, Soares P, Sobrinho-Simões M. The preeminence of growth pattern and invasiveness and the limited influence of BRAF and RAS mutations in the occurrence of papillary thyroid carcinoma lymph node metastases. Virchows Archiv. 2011;459(3):265-76.
33. Lloyd RV, Buehler D, Khanafshar E. Papillary thyroid carcinoma variants. Head and neck pathology. 2011;5(1):51-6.
34. Castro P, Rebocho A, Soares R, Magalhaes J, Roque L, Trovisco V, et al. PAX8-PPAR γ rearrangement is frequently detected in the follicular variant of papillary thyroid carcinoma. The Journal of Clinical Endocrinology & Metabolism. 2006;91(1):213-20.
35. Santoro M, Carlomagno F, Hay I, Herrmann M, Grieco M, Melillo R, et al. Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. Journal of Clinical Investigation. 1992;89(5):1517.
36. Lloyd RV, Erickson LA, Casey MB, Lam KY, Lohse CM, Asa SL, et al. Observer variation in the diagnosis of follicular variant of papillary thyroid carcinoma. The American journal of surgical pathology. 2004;28(10):1336-40.
37. Amacher AM, Goyal B, Lewis Jr JS, El-Mofty SK, Chernock RD. Prevalence of a Hobnail Pattern in Papillary, Poorly Differentiated, and Anaplastic Thyroid Carcinoma: A Possible Manifestation of High-grade Transformation. The American journal of surgical pathology. 2015;39(2):260-5.
38. Park JH, Kwon HJ, Park CS, Hong S. Anaplastic Transformation of Papillary Thyroid Carcinoma in a Young Man: A Case Study with Immunohistochemical and BRAF Analysis. Korean journal of pathology. 2014;48(3):234-40.

39. Cherkaoui GS, Guensi A, Taleb S, Idir MA, Touil N, Benmoussa R, et al. Poorly differentiated thyroid carcinoma: a retrospective clinicopathological study. The Pan African medical journal. 2015;21.
40. Soares P, Lima J, Preto A, Castro P, Vinagre J, Celestino R, et al. Genetic alterations in poorly differentiated and undifferentiated thyroid carcinomas. Current genomics. 2011;12(8):609.
41. Sobrinho-Simões M, Magalhães J, Soares P. Hot topics in papillary thyroid carcinoma. International journal of surgical pathology. 2010;18(3 suppl):190S-3S.
42. Sobrinho-Simões M, Preto A, Rocha AS, Castro P, Máximo V, Fonseca E, et al. Molecular pathology of well-differentiated thyroid carcinomas. Virchows Archiv. 2005;447(5):787-93.
43. Nakazawa T, Celestino R, Machado JC, Cameselle-Teijeiro JM, Vinagre J, Eloy C, et al. Cribriform-morular variant of papillary thyroid carcinoma displaying poorly differentiated features. International journal of surgical pathology. 2013:1066896912473355.
44. Cameselle-Teijeiro J, Abdulkader I, Pérez-Becerra R, Vázquez-Boquete A, Alberte-Lista L, Ruiz-Ponte C, et al. BRAF mutation in solid cell nest hyperplasia associated with papillary thyroid carcinoma. A precursor lesion? Human pathology. 2009;40(7):1029-35.
45. Fallahi P, Mazzi V, Vita R, Ferrari SM, Materazzi G, Galleri D, et al. New Therapies for Dedifferentiated Papillary Thyroid Cancer. International journal of molecular sciences. 2015;16(3):6153-82.
46. Nakazawa T, Murata S-i, Kondo T, Niu D, Mochizuki K, Kawasaki T, et al. RET/PTC rearrangements arising from a small population of papillary thyroid carcinoma cells, possible candidate for passenger mutation. Virchows Archiv. 2009;455(1):35-41.
47. Zhang T, Lu Y, Ye Q, Zhang M, Zheng L, Yin X, et al. An Evaluation an Recommendation of the Optimal methodologies to Detect Gene Rearrangements in Papillary Thyroid Carcinoma. Genes, Chromossomes & Cancer. 2014
48. Nikiforov YE. RET/PTC rearrangement in thyroid tumors. Endocrine pathology. 2002;13(1):3-16.
49. Brzeziańska E, Karbownik M, Migdalska-Sęk M, Pastuszak-Lewandoska D, Włoch J, Lewiński A. Molecular analysis of the RET and NTRK1 gene rearrangements in papillary thyroid carcinoma in the Polish population. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2006;599(1):26-35.

50. Marotta V, Guerra A, Sapio MR, Vitale M. RET/PTC rearrangement in benign and malignant thyroid diseases: a clinical standpoint. European Journal of Endocrinology. 2011;165(4):499-507.
51. Brose MS. In search of a real “targeted” therapy for thyroid cancer. Clinical Cancer Research. 2012;18(7):1827-9.
52. Reis-Filho JS, Preto A, Soares P, Ricardo S, Cameselle-Teijeiro J, Sobrinho-Simões M. p63 expression in solid cell nests of the thyroid: further evidence for a stem cell origin. Modern pathology. 2003;16(1):43-8.
53. Cameselle-Teijeiro J, Varela-Durán J, Sambade C, Villanueva JP, Varela-Núñez R, Sobrinho-Simoes M. Solid cell nests of the thyroid: light microscopy and immunohistochemical profile. Human pathology. 1994;25(7):684-93.
54. Kim SS LC, Bancroft JD. Theory and Practice of Histological Techniques: Churchill Livingstone
55. de Vries MM, Celestino R, Castro P, Eloy C, Máximo V, van der Wal JE, et al. RET/PTC rearrangement is prevalent in follicular Hürthle cell carcinomas. Histopathology. 2012;61(5):833-43.
56. Park G, Kim TH, Lee H-O, Lim JA, Won J-K, Min HS, et al. Standard immunohistochemistry efficiently screens for anaplastic lymphoma kinase rearrangements in differentiated thyroid cancer. Endocrine-related cancer. 2015;22(1):55-63.
57. Chou A, Fraser S, Toon CW, Clarkson A, Sioson L, Farzin M, et al. A Detailed Clinicopathologic Study of ALK-translocated Papillary Thyroid Carcinoma. The American journal of surgical pathology. 2015;39(5):652.

8 - ANNEXES

8 - ANNEXES

This work is submitted for publication:

- **Oliveira G**, Polónia A, Leitão D, Sapia S, Sobrinho-Simões M and Eloy C. *EWSR1* rearrangement is a frequent event in papillary thyroid carcinoma and in carcinoma of the thyroid with Ewing family tumour elements (CEFTE) (Submitted)